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Chapter

Systematic Deletion of Type III Secretion System Effectors in Enteropathogenic *E. coli* Unveils the Role of Non-LEE Effectors in A/E Lesion Formation

Massiel Cepeda-Molero, Stephanie Schüller, Gad Frankel and Luis Ángel Fernández

Abstract

Enteropathogenic *E. coli* (EPEC) is a diarrheagenic human pathogen. The hallmark of EPEC infection is the formation of the attaching and effacing (A/E) lesion in the intestinal epithelial cells, characterized by the effacement of brush border microvilli and the intimate bacterial attachment to the enterocyte in actin-rich pedestal-like structures. The locus of enterocyte effacement (LEE) in the EPEC genome encodes a type III protein secretion system (T3SS) that translocates multiple effector proteins into the host cell to subvert cellular functions for the benefit of the pathogen. These effectors are encoded both within and outside the LEE. In vitro cell culture infections have shown that LEE effectors are required for intimate bacterial attachment to the epithelial cells, whereas non-LEE effectors mostly play a role in modulating inflammation and cell apoptosis in the gut epithelium. We constructed a set of EPEC mutant strains harboring deletions in the complete repertoire of genes encoding T3SS effectors. Infection of human intestinal in vitro organ cultures (IVOC) with these mutant strains surprisingly revealed that non-LEE effectors are also needed to induce efficient A/E lesion formation in the intestinal mucosal tissue.

Keywords: A/E lesion, EPEC, effectors, infection, IVOC, T3SS

1. Introduction

1.1 Enteropathogenic *E. coli*

Enteropathogenic *E. coli* (EPEC) was the first pathotype of *E. coli* to be associated with human disease and is a major cause of acute and chronic diarrhea in infants [1, 2]. The low microbial density of the small bowel caused by the forceful peristalsis in this part of the intestine is overcome by EPEC, which can successfully colonize the small intestine of humans [3, 4]. EPEC primarily affects children younger than 2 years old; however some outbreaks of EPEC infection in healthy

adults have been associated with large inoculum ingestion [5]. The mechanism of transmission of EPEC is the fecal-oral route. In the 1940s and 1950s, EPEC was an important cause of diarrhea in developed countries with a mortality of 50% during outbreaks, but nowadays the infection by EPEC in industrial countries has a limited importance. In contrast, in low-income countries, EPEC is still an major cause of infant diarrhea [5, 6].

1.2 Hallmark of EPEC gastrointestinal infection

The phenotype that defines EPEC infection is the attaching and effacing (A/E) lesion [2, 7]. By adhering to intestinal epithelial cells, EPEC subverts cytoskeletal processes of the host cell and produces the histopathological feature of the A/E lesion. This lesion, which was first described in 1980 [8], is characterized by the intimate attachment of the bacteria to the intestinal epithelial cells and elongation and effacement of the brush border microvilli. Later on it was shown that infection is also associated with cytoskeletal rearrangements, including the accumulation of polymerized F-actin in pedestal-like structures underneath the attached bacteria [9] (**Figure 1**). EPEC together with enterohemorrhagic *E. coli* (EHEC) and *Citrobacter rodentium* (CR) is a member of the A/E family of bacterial pathogens that colonize the gastrointestinal tract via the A/E lesion. EPEC and EHEC are important human pathogens, while CR is a mouse-restricted pathogen [10–13].

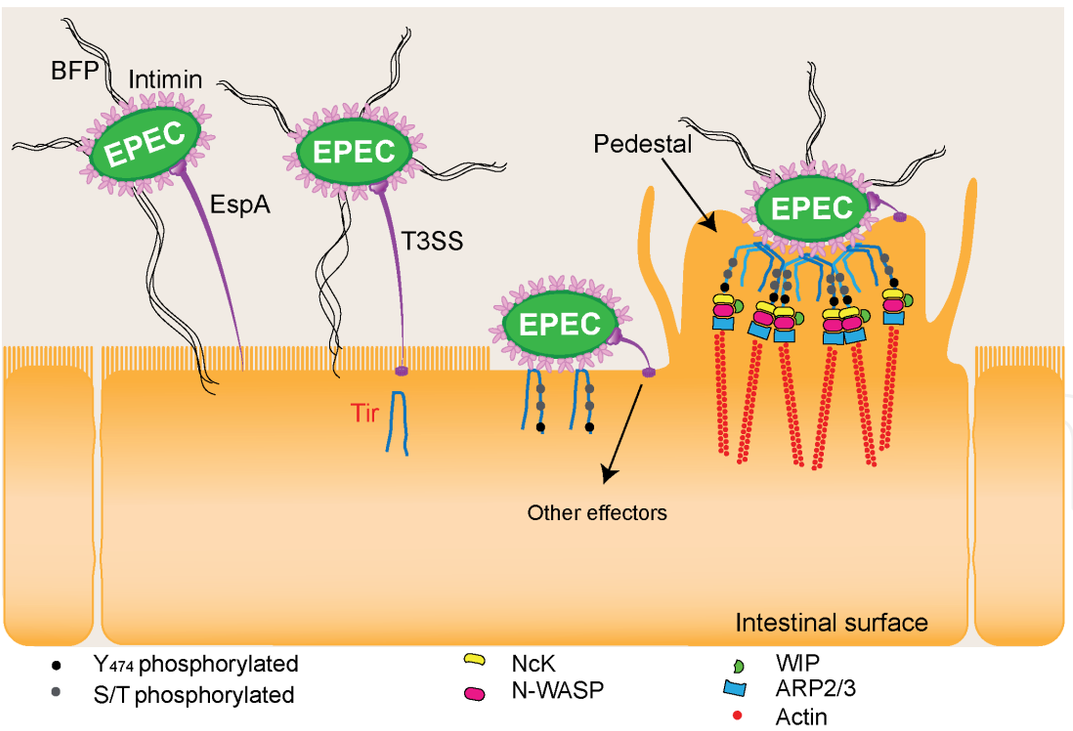

Figure 1.
Localized adherence (LA), intimate attachment, and EPEC A/E lesion formation in the intestinal epithelial surface. At an early stage, EPEC interacts in a non-intimate manner with the intestinal surface mainly through the BFP and EspA filament. After assembly of the translocation pore, EPEC injects translocated intimin receptor (Tir). Ser/Thr phosphorylation of Tir induces its anchoring in the enterocyte plasma membrane, leaving the TirM region exposed for the interaction with intimin. Subsequent Tir-intimin interaction triggers actin polymerization and pedestal formation underneath the attached bacterium. Tir phosphorylation of residue Y474 engages the host adaptor NcK, which later recruits N-WASP and WIP. N-WASP recruits the ARP2/3 complex, which induces actin nucleation and polymerization.

2. EPEC virulence factors

2.1 A pathogenicity island called LEE

The ability of EPEC to induce A/E lesions is related to a pathogenicity island (PAI) of 35 kb called the locus of enterocyte effacement (LEE) [14]. The LEE comprises 41 genes organized in 5 principal operons (LEE1-LEE5) and several smaller transcriptional units (**Figure 2**) [15, 16]. Orthologues of LEE are also found in other members of A/E pathogens [11]. The LEE encodes all the structural proteins necessary for the assembly of a filamentous type III secretion system (T3SS) injectisome on the bacterial cell envelope [17, 18]. The LEE also encodes transcriptional regulators (Ler, GrlR, and GrlA), translocator proteins (EspA, EspB, and EspD), six secreted effector proteins (including the translocated intimin receptor), the outer membrane protein intimin, molecular chaperones, and a lytic transglycosylase (EtgA) [19]. The mechanism of LEE regulation is complex and depends on environmental conditions, quorum sensing (QS), and several transcriptional regulators encoded within and outside the LEE [20, 21].

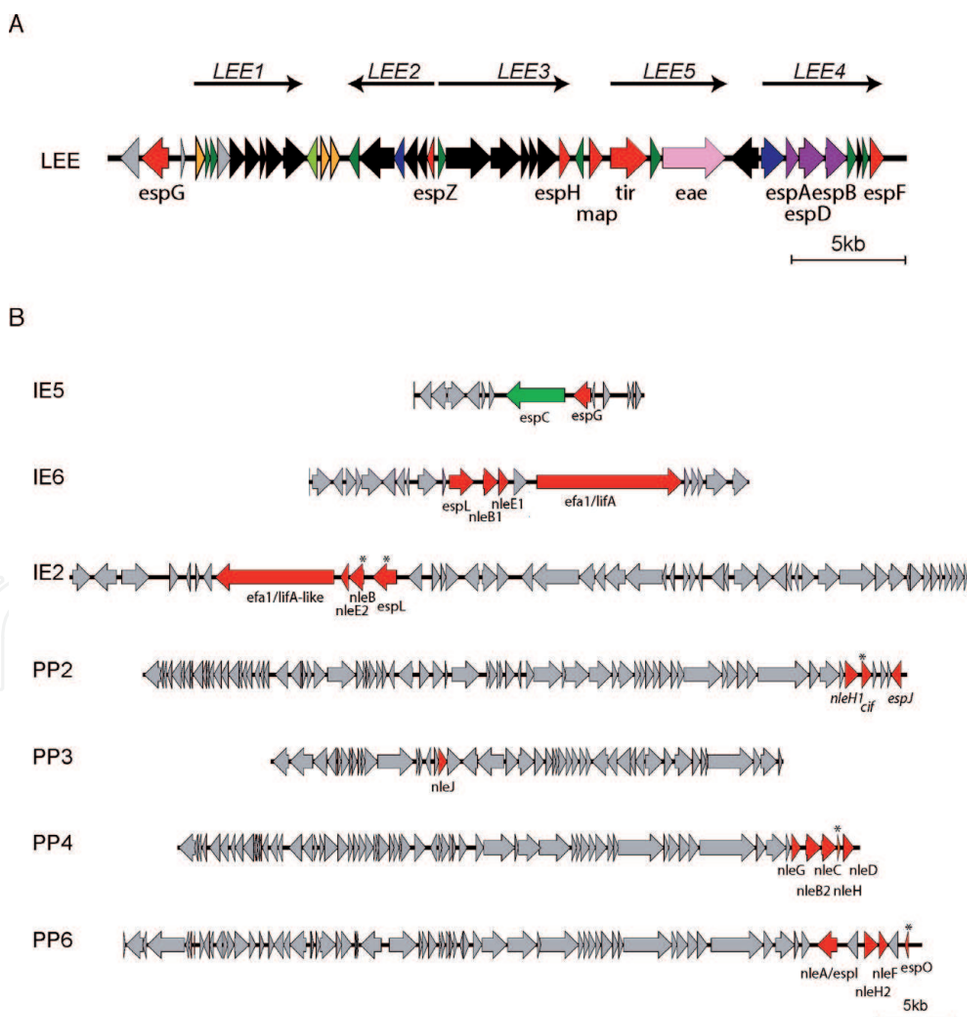

Figure 2.
Effectors of EPEC E2348/69. (A) Representation of the LEE island and effector genes espG, espZ, espH, map, tir, and espF. (B) Non-LEE effectors located outside the LEE are localized in integrative elements (IEs) and prophages (PPs). Effector genes are labeled in red. Pseudogenes are specified with asterisk. Scale of 5 kb is indicated at the bottom. Figure from [32].

2.2 The type III secretion system

The type III secretion system is a macromolecular transport apparatus that is used by many gram-negative bacterial pathogens (e.g., *Shigella*, *Yersinia*, *Salmonella*) to translocate virulence proteins, called effectors, into the cytosol of infected cells, thereby subverting host cellular functions for the benefit of the pathogen [22]. Since pathogens use this transport apparatus to inject proteins into the host cells, this structure is also known as the injectisome. The EPEC T3SS mediates the translocation of multiple effector proteins during infection. Some of them are encoded in the LEE, whereas others are encoded outside of the LEE being generally referred to as non-LEE effectors (Nle) [23, 24]. EspA filaments link the tip of the injectisome in the bacterial cell wall to a 3–5 nm translocation pore, formed in the plasma membrane of infected cells by the translocator proteins EspB and EspD (**Figure 1**) [25, 26].

2.3 Bundle-forming pilus (BFP)

Typical EPEC is endowed with a plasmid called pMAR2 which contains a 14-gene operon encoding the type IV pilus BFP [27, 28]. The BFP is a rope-like bundle, which allows EPEC to form microcolonies in a pattern called localized adherence and also mediates the initial interaction of bacteria with host cell surfaces (**Figure 1**) [29–31].

3. EPEC pathogenesis

EPEC tightly regulates its virulence genes in response to environmental conditions such as temperature [16], the increase of the pH of the small intestine [33, 34], and some hormones which are released during stress conditions [20]. Upon EPEC interaction with enterocytes, EspB and EspD proteins are inserted into the host cell membrane and assemble to form a translocation pore [25, 26]. EPEC then injects its own receptor called Tir, which is integrated into the plasma membrane in a hairpin loop topology, with the loop facing the outside of the cell where it serves as a receptor for the bacterial adhesin intimin [35–37]. Tir-intimin interaction induces clustering and dimerization of Tir, and this activates a signal cascade that starts with the phosphorylation of serine/threonine residues and leads to actin polymerization and pedestal formation underneath the attached bacterium [10, 38]. The most critical event for actin polymerization is the phosphorylation of the cytoplasmic Tir residue Y474 [39]. This induces a signal cascade which recruits the host cell adaptor Nck and N-WASP required to engage and activate the actin-nucleating ARP2/3 complex, which produces the actin nucleation and polymerization. Actin polymerization drives membrane protrusion and pedestal formation [10, 40] (**Figure 1**). Through the T3SS injectisome, EPEC translocates LEE-encoded effector proteins and additional effectors localized in mobile genetic element outside the LEE (Nle).

4. LEE effectors

Six effector proteins (EspG, EspZ, EspH, Map, Tir, and EspF) are encoded in the LEE island (**Figure 2**). Most of these, except EspZ, have important functions destabilizing the physiology of the intestinal epithelium, triggering cytoskeleton reorganization, inducing cytotoxicity and electrolyte imbalance which lead to

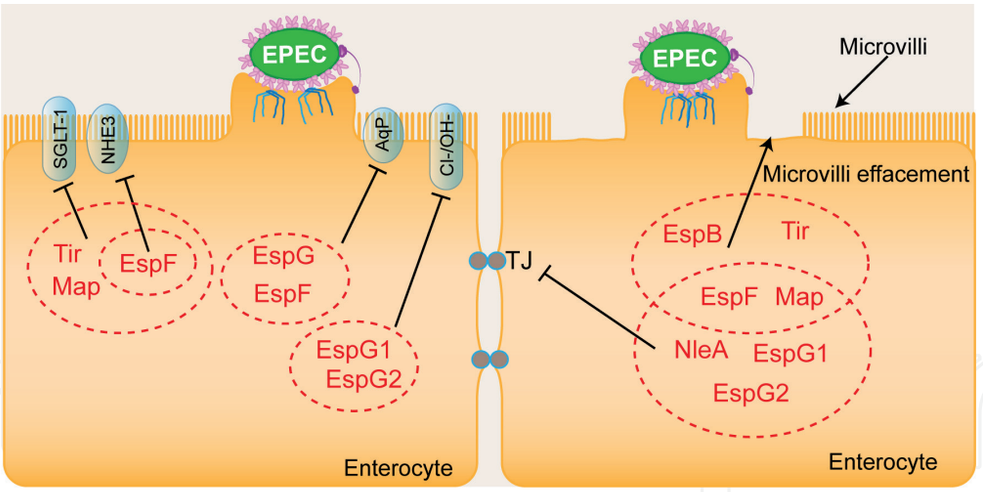

Figure 3.
EPEC effector proteins altering epithelial cell function and inducing water loss and diarrhea. Tir, map, and EspF inhibit the sodium-D-glucose transporter. EspF reduces expression of the sodium-hydrogen exchanger NHE3. EspG and EspF induce mislocalization of aquaporins (AqP). EspG1/EspG2 alters membrane targeting of the Cl-/OH-exchanger. EspF, map, NleA, EspG1, and EspG2 disrupt tight junction complexes (TJ). EspB, Tir, EspF, and map induce microvilli effacement.

diarrhea [11, 41]. The rapid onset of EPEC-induced diarrhea is likely induced by the cooperative action of Tir, Map, and EspF, which inhibits the sodium-D-glucose transporter (SGLT-1), the major water pump of the small intestine responsible for about 70% of the total fluid uptake [42]. In addition, Map and EspF reduce Na + absorption by the sodium-hydrogen exchanger (NHE3) [43], and EspG1/2 proteins alter the membrane targeting of the Cl-/OH-exchanger (DRA), resulting in reduced Cl-uptake. These processes result in the accumulation of salts in the gut lumen, which drives water loss from the mucosa [44].

Inhibition of endosomal trafficking by EspG1/2 reduces the level of cell surface receptors [45]. In addition, EspF and EspG induce mislocalization of aquaporins (AQP), thereby reducing epithelial water absorption [46]. Furthermore, EspB, Tir, EspF, and Map induce microvillus effacement, and this reduction of absorptive surface likely exacerbates EPEC diarrhea [47]. While EspF and Map synergistically disrupt TJs [48], EspG1/2 induces microtubule disruption contributing to TJ disruption [49]. The effector protein NleA also disrupts TJs by blocking the delivery of new TJ proteins [49–51]. The disruption of TJs increases intestinal permeability and thereby likely contributes to EPEC-induced diarrhea [52] (**Figure 3**).

5. Non-LEE effectors

In EPEC prototype strain E2348/69, 17 functional Nle effectors are encoded in different integrative elements and prophages, frequently associated in gene clusters, with some effectors having duplicated gene copies and/or paralogs in different clusters [53] (**Figure 2**). EPEC infection is characterized by a weak inflammatory response [54]. Previous studies have shown that most Nle effectors and some LEE effectors inhibit the host immune response, which favors bacterial survival (**Figure 4**). Although NleF and NleH2 activate the NF-κB inflammatory pathway during early infection (ref), EPEC translocates several effectors that dampen the proinflammatory pathways of the cell [11]. Thus, a large number of Nle effectors inhibit host inflammation by different mechanisms, such as inhibition of the NF-κB (NleB, C, E, and H) and MAPK proinflammatory pathways (NleC and D) [55–58], inhibition

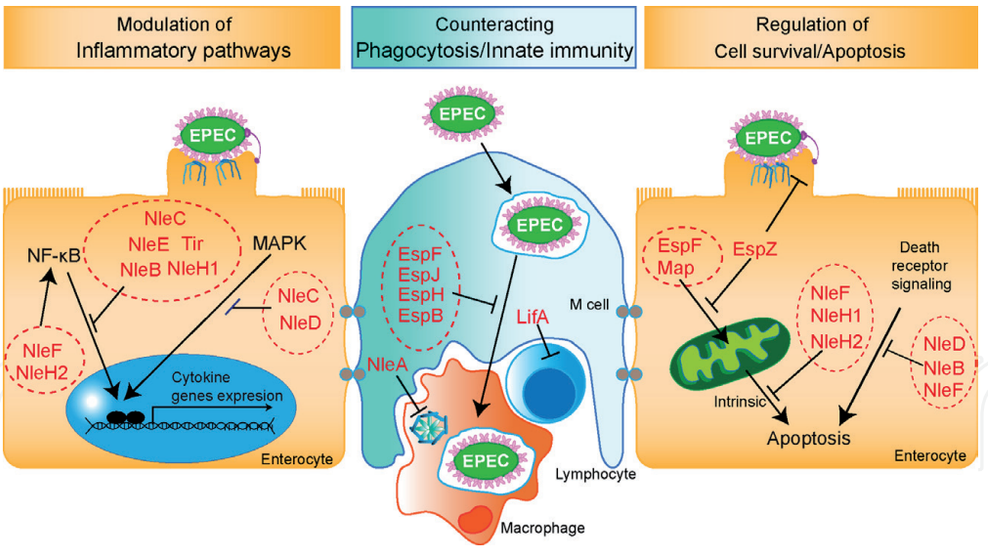

Figure 4.
Schematic representation of multifunctional and overlapping effectors to control host immune response. The NF-κB proinflammatory pathway is activated by NleF and NleH2 and is inhibited by NleE, NleB, NleH1, Tir, and NleC. NleC and NleD inhibit the MAPK proinflammatory pathway. EspF, EspJ, EspH, and EspB prevent macrophage phagocytosis. NleA disrupts inflammasome activation, and LifA inhibits IL-2 and IL-4 production and lymphocyte proliferation. While EspF and map induce intrinsic apoptosis, EspZ counteracts these effects by stabilizing mitochondrial membrane potential. NleH1/NleH2 and NleF inhibit intrinsic apoptosis, and NleF, NleD, and NleB counteract extrinsic apoptosis.

of the canonical (NleA) and noncanonical (NleF) inflammasomes [59], and inhibition of proliferation of lymphocytes and interleukin production (LifA) [60, 61].

The control of the epithelial cell death response to microbial infection is pivotal for pathogens and the host. Pathogens that are colonizing the epithelium need to prevent cell death to preserve their replicative foothold; by contrast, the host needs to eliminate infected cells in order to minimize tissue damage [62]. During infection of the intestinal epithelial cells, surface properties of EPEC are recognized by cell surface death receptors and induce extrinsic apoptotic pathways, while T3SS effectors (Map and EspF) trigger cytochrome c release, activation of caspases, and downstream intrinsic apoptotic pathways [11, 24]. Interestingly, early stages of apoptosis can be observed during EPEC infection, but late stages are not evident because EPEC translocates effector proteins that antagonize these pro-apoptotic effects. NleD and NleB interfere with the pro-apoptotic death receptor signaling and disrupt the downstream extrinsic apoptosis [63, 64]. NLeH1/2 and EspZ also inhibit intrinsic apoptosis and promote host cell survival [65–67] (**Figure 4**). NleF directly inhibits caspases involved in both intrinsic and extrinsic apoptosis pathways, including caspases 4, 8, and 9 [68]. In addition, EspZ localizes to the cytoplasmic side of the plasma membrane at the site of bacterial attachment and interacts with the translocator protein EspD. It has been proposed that EspZ indirectly prevents cell death by downregulating protein translocation and protecting cells from an overdose of effector proteins. Consistently, a Δ*espZ* mutant was found to be highly cytotoxic [69]. EPEC effectors are injected in a regulated manner to guarantee the success of infection. While the pro-survival effector EspZ is translocated at the early stages of infection, the pro-apoptotic effectors EspF and Map follow later [70].

6. Classical methodologies to study effector functions

Most research on EPEC effectors has been conducted by generating deletion mutants in a single or a few effector genes that are later complemented with

multicopy plasmids overexpressing the effector(s). In addition, ectopic expression of individual effectors by plasmid transfection of the host cell has been applied. Both situations are prone to effector overexpression resulting in nonphysiological levels of effectors inside the host cell, which could alter effector activities. In addition, effectors often have synergistic and overlapping functions that cannot be fully appreciated by single mutations and individual transfection experiments [11, 54]. In order to overcome these limitations, we employed a marker-less gene deletion strategy to delete the whole repertoire of known effector genes found in the genome of the prototypical EPEC strain E2348/69 [32]. The genome engineering method for sequential deletion of EPEC effectors was based on the marker-less gene deletion technique described by Posfai et al. [71] and is illustrated in **Figure 5**.

Using this strategy, a set of EPEC mutants with sequential deletions of effectors was generated (**Table 1**), ultimately resulting in strains expressing only Tir and EspZ (EPEC2), Tir (EPEC1), and the effector-less strain EPEC0 (**Table 1**). This approach proved to be effective to specifically modify the genome of EPEC E2348/69, avoiding the introduction of unintended alterations in the genome and leaving no sequence "scars" or antibiotic resistance genes in the chromosome as demonstrated by whole-genome sequencing [32]. Besides, the deletion mutant strains showed normal growth and maintained functional T3SS injectisomes. In addition, they allowed the translocation of individual effectors from single-copy chromosomal genes under endogenous regulation, showing the expected phenotypes without the background of the other effectors [32]. Hence these mutant

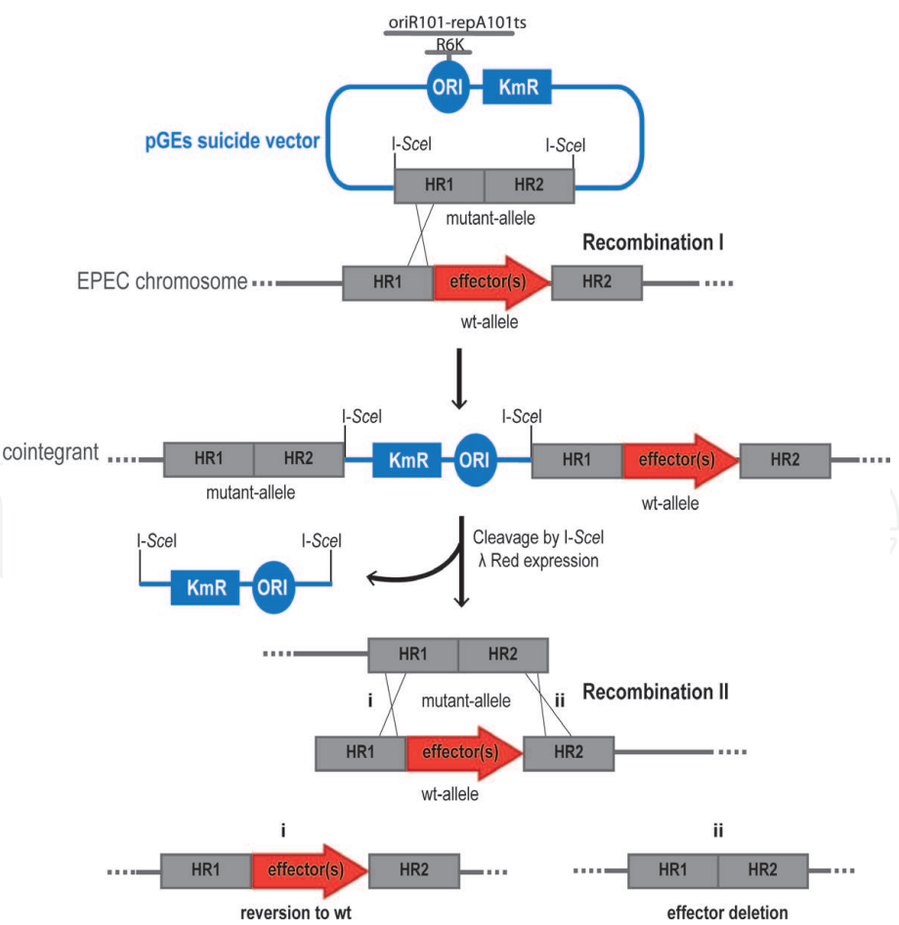

Figure 5.
Marker-less gene deletion strategy of EPEC effector genes. Deletions using pGE-suicide plasmids with I-SceI sites and mutant alleles assembled by fusing homology regions (HRs) flanking the targeted effector gene(s). Co-integrants are identified by the Kanamycin resistance phenotype. Expression of the I-SceI in vivo from helper plasmid induces double-strand brakes that are repaired by homologous recombination. Depending on the HRs involved in this second recombination, either the WT or the mutant allele can be obtained. Figure from [32].

Strain	Effector genes remaining*
WT	All
EPEC2	*espZ* and *tir*
EPEC1	*tir*
EPEC0	None
EPEC9	*espZ + tir + IE2 + IE5 + IE6 + PP2 + PP3 + PP4 + PP6*
EPEC2-LEE+	*espZ + tir + map + espH + espF + espG*

**Encoded effectors in the indicated IEs and PPs.*

Table 1.
EPEC mutant strains generated with the marker-less deletion strategy.

strains are an excellent tool to investigate the role of individual effectors and specific combinations maintaining physiological protein levels in the context of infection.

7. LEE effectors are sufficient for intimate adhesion of EPEC to the epithelial cells in vitro

When EPEC bacteria adhere in vitro to cultured cells, there is an accumulation of actin filaments in the cytoplasm beneath the adherent bacteria, due to a signal cascade triggered by intimin-Tir interaction [35, 38]. Using the effector deletion

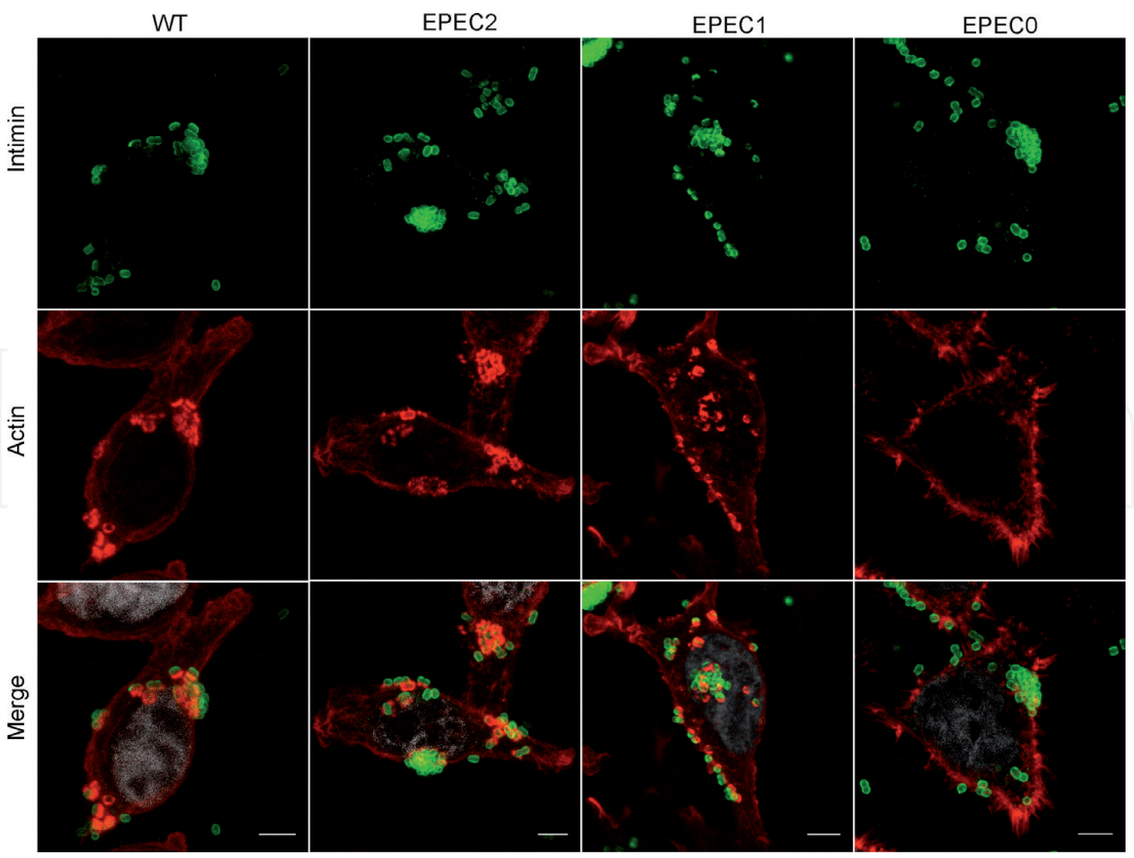

Figure 6.
Infection of HeLa cells with EPEC WT and effector mutant strains. Immunofluorescence confocal microscopy of HeLa cells infected with EPEC WT, EPEC2, EPEC1, and EPEC0 for 1.5 h using a MOI of 200. EPEC is labeled with anti-intimin-280 serum (green), actin is stained with TRITC phalloidin (red), and cell nuclei are labeled with DAPI (gray). Actin polymerization beneath adherent bacteria is observed in EPEC WT, EPEC2, and EPEC1. Scale bar 5 μm. Figure from [32].

mutants of EPEC, we demonstrated that the LEE effector Tir along with intimin is necessary and sufficient to induce these cytoskeletal rearrangements during in vitro infection of HeLa cells. Strains EPEC2 (bearing EspZ and Tir) and EPEC1 (bearing only Tir) were able to induce actin-pedestal formation underneath attached bacteria similar to the EPEC wild type (WT) (**Figure 6**). As expected because of the essential role of Tir in this process, infection of HeLa cells with the effector-less mutant EPEC0 did not induce any actin-pedestal formation (**Figure 6**). These data demonstrate that the individual translocation of Tir by EPEC1 is sufficient to trigger actin pedestals in HeLa cells and that non-LEE effectors are dispensable for this phenotype during in vitro infection of cultured cells.

8. Non-LEE effectors are required for efficient A/E lesion formation in intestinal tissue

EPEC pathogenic mechanisms have been widely investigated by in vitro infection of cultured epithelial cell lines, albeit in most cases these cells are non-polarized and are not from intestinal origin. In addition, EPEC infection studies in vivo are hindered because EPEC is a human-restricted pathogen [72]. A surrogate model established to investigate A/E pathogenesis in vivo is the mouse pathogen *Citrobacter rodentium* (CR) [12, 13]. Although *Citrobacter* infection in vitro requires Tir phosphorylation for actin-pedestal formation in cell lines, Tir phosphorylation-deficient mutants still colonize the mouse gut and induce A/E lesion formation and crypt hyperplasia typical of CR infection [73]. This result highlights the necessity of a model for EPEC infection closer to the in vivo conditions in the human gut. A good established model to study EPEC-host interactions is the infection of in vitro cultured human intestinal biopsies, which allows the formation of A/E lesions undistinguishable from those observed in vivo in biopsies of patients with EPEC-induced diarrhea [4, 36, 74]. Similar to results obtained in CR-infected mice, Tir phosphorylation was not necessary for EPEC A/E lesion formation in human intestinal biopsies [75]. Surprisingly, when EPEC2 and EPEC1 deletion mutants were used to infect human duodenal biopsies, none of the infected biopsies showed A/E lesions (**Table 2** and **Figure 7**), which contrasts with the pedestal formation observed in HeLa cells. Thus, intimin and Tir are not sufficient to induce A/E lesions in the intestinal tissue, and the IVOC model was used to identify additional LEE or non-LEE effector(s) required for A/E lesion formation. For this purpose, two additional effector mutant strains were tested: EPEC2-LEE+ (carrying all LEE effectors) and EPEC9 (carrying EspZ, Tir, and

Strain	Effector genes remaining*	Biopsies with A/E lesions positive/total (%)
WT	All	13/17 (76)
EPEC2	*espZ* and *tir*	0/6 (0)
EPEC1	*tir*	0/6 (0)
EPEC0	None	0/5 (0)
EPEC9	*espZ + tir + IE2 + IE5 + IE6 + PP2 + PP3 + PP4 + PP6*	5/6 (83)
EPEC2-LEE+	*espZ + tir + map + espH + espF + espG*	0/5 (0)

**Encoded effectors in the indicated IEs and PPs.*

Table 2.
Human duodenal biopsies infected by EPEC WT and EPEC effector mutants.

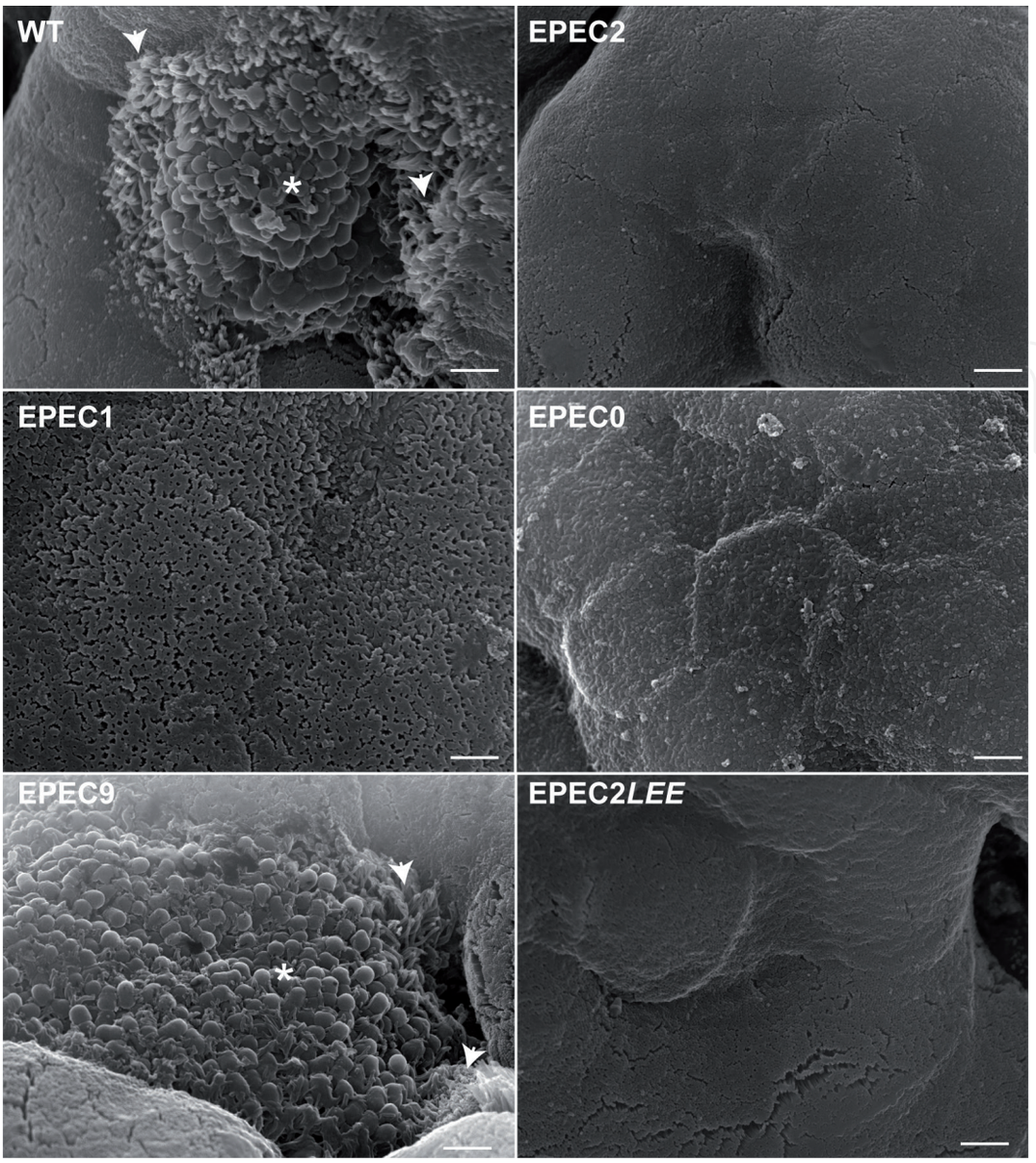

Figure 7.
Scanning electron microscopy of human duodenal biopsies infected with EPEC WT and mutant strains EPEC2, EPEC1, EPEC0, EPEC9, and EPEC2-LEE+. EPEC WT and EPEC9 induce characteristic A/E lesions with bacterial microcolony formation (asterisk) and microvilli elongation around bacterial colonies (arrowheads). In contrast, biopsies infected with EPEC2, EPEC1, EPEC0, and EPEC2-LEE+ lack adherent bacteria and A/E lesions and show a normal microvillous brush border. Scale bar 2 μm. Figure from [32].

all non-LEE effectors). Whereas infection with EPEC2-LEE+ did not reveal A/E lesions, infection with EPEC9 induced A/E lesions to a similar level as the wild-type strain (**Table 2** and **Figure 7**). It was previously reported that the LEE island is sufficient to confer the A/E phenotype to *E. coli* K-12 in the infection of colon carcinoma cell lines [76]. However, our results indicate that the LEE is not sufficient for A/E lesion formation in human mucosal tissue and that non-LEE effectors are required [32].

9. Conclusions and future perspectives

The marker-less gene deletion strategy enabled the generation of effector-less strains of EPEC O127:H6 using the prototypical strain E2348/69 [32]. Given the conservation of the recombination machinery among *E. coli* strains, it is likely that

this strategy could be applied to other A/E pathogens, *E. coli* pathogens, and other bacteria. The effector mutant strains can be useful to study the role of individual effectors and of combinations of effectors in pathogenesis. An individual effector or a defined combination can be inserted in the effector-less strains in their endogenous genomic loci to obtain physiological expression levels and regulation. In cell culture infections, all EPEC effector mutant strains carrying intimin and Tir were able to trigger actin-rich pedestal-like structures underneath attached bacteria. On the other hand, when the infection was performed in human intestinal tissues, translocation of Tir alone was insufficient to induce A/E lesions. Furthermore, an EPEC deletion mutant maintaining all LEE effectors and devoid of all non-LEE effectors (EPEC2-LEE+) was still unable to induce A/E lesions in human intestinal biopsies. In contrast, an EPEC strain producing the complete repertoire of non-LEE effectors and devoid of LEE effectors, except Tir and EspZ, formed A/E lesions in intestinal tissue at wild-type levels [32]. Thus, these experiments revealed that non-LEE effectors are needed for A/E lesion formation in human intestinal tissue.

In addition to their potential for basic studies, the EPEC effector mutant strains may have different applications. For instance, EPEC (and other pathogenic) strains lacking multiple effectors are likely to be strongly attenuated, but they maintain the external antigenicity of the wild-type strain. Thus, an EPEC mutant strain with a functional T3SS and the minimum set of effectors necessary to colonize the intestinal surface could be a good vaccine candidate. Further, EPEC mutant strains with the ability to attach to the human intestine could also be engineered to translocate heterologous protein antigens to generate protection against other enteric pathogens causing diarrhea, including EHEC strains [77–80]. Lastly, the EPEC effector mutant strains may also have the potential to deliver therapeutic proteins to the intestinal epithelium, for instance, to combat inflammation and autoimmune disorders in the gastrointestinal tract [81].

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